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14. ABSTRACT

The objective of this project is to discover and characterize molecules that inhibit breast cancer cell proliferation by maintaining activity of the retinoblastoma protein (Rb). Rb is inactivated to drive proliferation in normal and cancer cells by phosphorylation, which dissociates the E2F transcription factor from Rb. Our goal is to find and characterize molecules that stabilize the complex between phosphorylated Rb and E2F.

In this second year of the project period, we further tested our proposed mechanism for how molecules enhance the affinity of phosphorylated Rb for E2F by disrupting the compact Rb conformation. We performed experiments to validate the efficacy of compounds identified as hits in our primary screen, and we began pursuing additional approaches to identifying lead compounds.

15. SUBJECT TERMS
cell cycle, Retinoblastoma protein, E2F transcription factor, high throughput screen, drug discovery, x-ray crystallography

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1.0 INTRODUCTION

The Rb pathway connects environmental and intracellular growth signals to the cell cycle machinery that drives cell division. Inactivation of Rb pathway function is found in most human cancers, including breast cancer. Inhibition of cell proliferation by Rb is linked to its direct binding of E2F transcription factors and repression of E2F activity. Rb inactivation occurs upon Cdk phosphorylation, which induces E2F release and activation of S phase genes. *Our overarching hypothesis is that we can modulate Rb activity directly with small molecules that inhibit or stabilize its association with E2F and thus control cell proliferation.* This project aims to identify such molecules with high-throughput screening, to validate hits in secondary and cellular assays, and to characterize the mechanism of lead compound interaction with Rb. This past year, the second year of the project, we further tested the hypothesized mechanism by which Rb-E2F affinity can be enhanced, examined a set of 108 hit compounds identified in the primary screen, and developed a method to perform fragment based screening by x-ray crystallography.

2.0 KEYWORDS

Retinoblastoma (Rb) pathway, E2F transcription factor, cancer, cell-cycle inhibition, activation, modulation, inhibition, high throughput screening, fragment-based screening, x-ray crystallography.

3.0 ACCOMPLISHMENTS

Summary:

We continued to test the hypothesis that molecules can be identified that enhance the affinity of phosphorylated Rb for E2F, and we further tested our proposed mechanism for how this may occur. Our results in these experiments and the results of our pilot screen were published in ACS Chemical Biology this year. We performed experiments to validate the efficacy of compounds identified as hits in our primary screen, but we found that they do not robustly enhance Rb-E2F affinity, and we found no evidence that they bind Rb. We began pursuing additional approaches to identifying lead compounds, including fragment-based screening and the screening of cyclic peptide libraries.

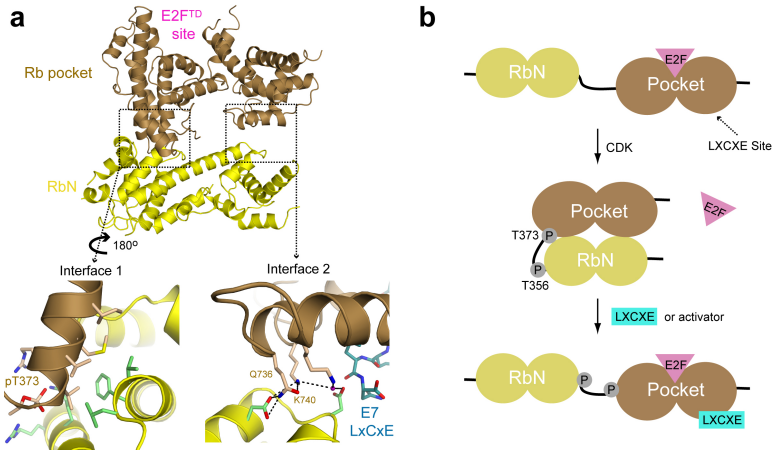

Figure 1. Model for activation of Rb. (a) Structure of phosphorylated Rb. Docking between the Rb N-terminal domain (RbN, yellow) and the pocket domain (brown) occurs across two interfaces. Interface 1 is mediated by T373 phosphorylation. Interface 2 is near the LxCxE-binding cleft in the pocket. The E7 peptide (cyan backbone), which is shown bound at its site in the unphosphorylated pocket, clashes with RbN residues at the interface. (b) Phosphorylation of sites in the Rb interdomain linker induces a conformational change that allosterically inhibits E2F binding. We find that an LxCxE peptide and a protein containing that sequence acts as an activator by binding Rb and inhibiting the RbN-pocket interdomain association.

Goals:

1) Test model for how compounds can enhance Rb-E2F affinity by disrupting the compact conformation of phosphorylated Rb.
2) Validate hits compounds from primary screen
2) Develop tools and protocols for fragment based screening

Detailed Accomplishments:

Task 1: Test hypothesis that disrupting phosphorylation-induced Rb conformational changes enhances

E2F binding

In the past year, we further explored our strategy for developing Rb activators. This approach is motivated by the structural mechanism underlying how the Rb-E2F complex is inhibited by Rb phosphorylation (Figure 1). Our previous work demonstrated that T373 phosphorylation in Rb induces an interdomain association between the Rb N-terminal domain (RbN) and the so-called pocket domain, which allosterically opens the E2F binding cleft in the pocket to weaken affinity. RbN-pocket association occurs across two interfaces (Figure 1a), both of which must be formed to open up the E2F-binding site and disrupt interactions between the pocket and E2F. The RbN position at one interface is close to the "LxCxE binding" site in the pocket domain. This cleft is a well characterized binding site for viral and cellular proteins that contain an LxCxE amino acid sequence. From a structural alignment (Figure 1a), the binding of the LxCxE peptide and interdomain docking appear incompatible. We reasoned that molecules that inhibit interdomain docking would stabilize E2F binding to phosphorylated Rb by preventing the allosteric opening of the E2F binding site (Figure 1b). Moreover, because the LxCxE peptide binds near the second RbN-pocket interface (Interface 2 in Figure 1a), we hypothesized that it would disrupt docking and act as an activator. We tested the effects of the LxCxE peptide from the HPV E7 protein on E2F binding to Rb using a fluorescence polarization (FP) assay (Figure 2). The affinity of E2F for unphosphorylated Rb (Kd = 4.3 ± 0.2 nM) is 8-fold tighter than its affinity for phosphorylated Rb in this assay (Kd = 31 ± 4 nM). In the presence of 10 μM E7 LxCxE peptide, phosphorylated Rb binds E2F with 2-fold higher affinity (Kd = 16 ± 2 nM), which implicates the LxCxE peptide as an example of a desired Rb activator molecule. In the presence of 2 μM full length E7 protein, E2F binds phosphorylated Rb with similar affinity (Kd = 4.6 ± 0.3 nM) as unphosphorylated Rb.

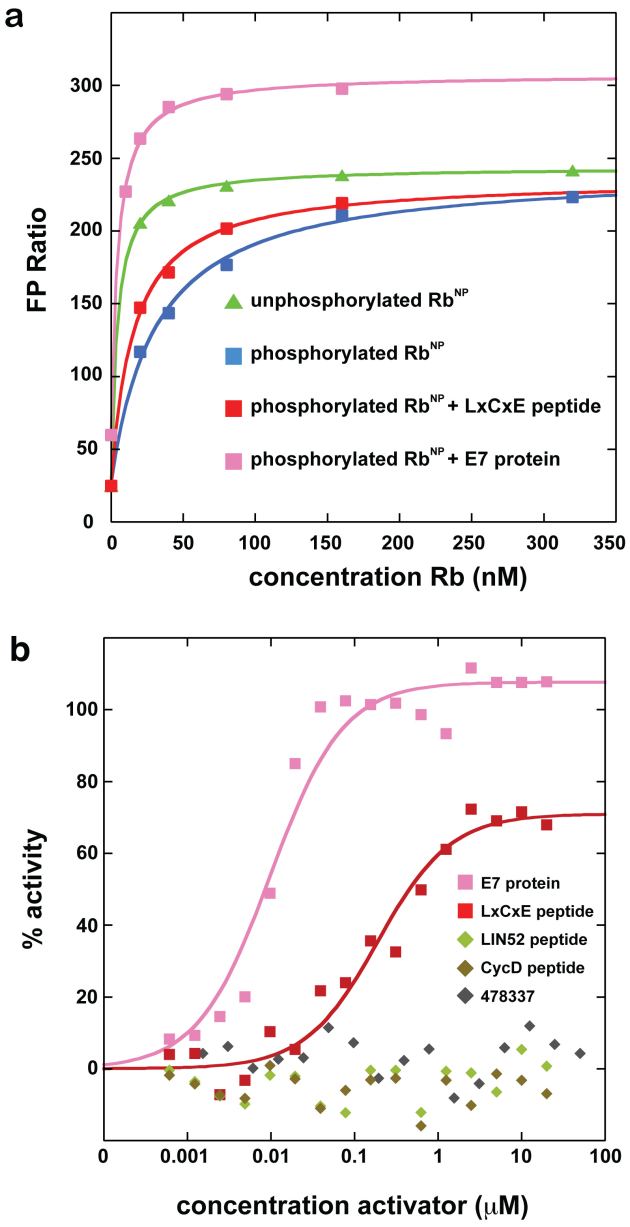

Figure 2. LxCxE peptide acts as an Rb activator. (a) Titration of unphosphorylated and phosphorylated Rb into E2F. In the presence of the E7 LxCxE peptide and full-length E7 protein (pink), the affinity is increased. (b) EC50 measurement of LxCxE peptide and E7 protein activity. Compound #478337) and LxCxE variant peptides from Cyclin D and LIN52 do not show activity.

We found that the LxCxE peptide and full-length E7 increased RbNP-E2F affinity with EC50 = 190 ± 40 nM and EC50 = 10 ± 2 nM respectively (Figure 2b). The greater potency of the full-length protein correlates with its known 20-fold greater affinity for the Rb pocket domain. We suggest that the greater highest activity of the protein (~100%) compared to the peptide (~70%) may result from the fact that its larger size is better suited for occluding the RbN-pocket interface. We found that the LxCxE-peptide and E7 protein do not affect E2F binding to Rb if the docking interface is mutated (Q736A/K740A), which supports further our proposal that these activators function by disrupting interdomain docking. We also tested two LxCxE-like peptides from

Cyclin D (no hydrophobic in +2 position) and LIN52 (LxSxE), which have weak affinity for Rb. These variant peptides and a compound previously reported to bind the LxCxE cleft (compound #478337 show no effect in the Rb activation assay (Figure 2b).

Task 2: Validate hits from primary screen and prepare reagents for a cyclic peptide library screen

In the first year of our funding period, we completed a screen of a large small molecule library (~320,000 compounds) at the Conrad Prebys Center for Chemical Genomics (CPCCG, at Sanford Burnham Institute). From the screen we identified 108 compounds as hits that reproduced in a dose response curve after cherry-picking the primary screen plates. This list was pared down to 40 top candidates based on chemoinformatics input from CPCCG. This past year at UCSC, we attempted to validate these hits by ordering fresh, dry compounds and testing them in the same FP assay. We found that about half of the 40 compounds did not repeat in the assay and the other half induced changes to the E2F FP signal even in the absence of Rb target. We conclude that these compounds are aggregating the E2F probe peptide and are not suitable lead compounds.

Based on these results, we decided not to pursue any hits from the initial small molecule library screen and instead focus on screening larger molecules that may be better suited for modulating protein-protein interactions. We successfully prepared the required protein to screen cyclic peptide libraries in collaboration with Professor Scott Lokey at UCSC.

Task 3: Crystallize Rb and E2F constructs for fragment based screening

As an additional approach to finding molecules that modulate Rb-E2F binding in the FP or similar assay, we are developing a fragment-based approach to identify molecules that bind either Rb or E2F. Once we identify fragments that bind these proteins, we will synthetically build molecules that are potentially capable of modulating the complex affinity. We are taking an x-ray crystallography approach in which a small fragment library will be screened by soaking compounds into crystals of Rb or E2F. Following x-ray data collection, new electron density corresponding to the fragments will be observable if there is binding, and the structural data can be used to develop more potent binders. This approach requires crystal forms of Rb and E2F that diffract well and are highly reproducible. From past work, we have such a crystal form of the Rb pocket domain, and this past year we focused on producing a suitable crystal of E2F. We were able to successfully grow crystals of the E2F4 marked-box and coiled-coiled domains (Figure 3), which are the primary structured regions of the E2F transcription factor. These crystals diffract to 1.8 angstroms and will be an excellent reagent for our forthcoming fragment screen.

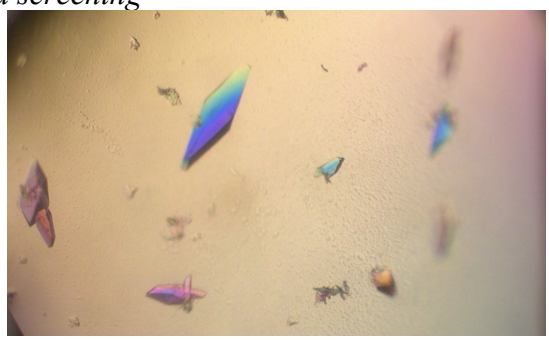

Figure 3. Crystals grown for E2F4 that diffract to 1.8 angstroms.

Training Opportunities: Nothing to report

Dissemination of Results:

Publication

Pye C.R., Bray W.M., Brown E.R., Burke J.R., Lokey R.S., Rubin S.M. A strategy for direct chemical activation of the retinoblastoma protein. ACS Chem Biol. 2016 May 20;11(5):1192-7.

Future plans:

We will perform the FP screen using a cyclic peptide library and pursue screening a fragment library

using x-ray diffraction. Once we have valid hit compounds, we will look at activity in influencing the cell cycle and proliferation of breast cancer cells. We will also make biophysical measurements of compound affinity for Rb and determine how E2F binding is modulated.

4.0 IMPACT

Our research is having impact by establishing an innovative approach to breast cancer therapeutic development. Most approaches to date for preventing cancer cell cycle progression have focused on upstream Cdk inhibitors. This approach is traditional because enzyme active sites are easily blocked with small molecules. We are proposing a transformative approach that restores tumor suppressor function in the presence of upregulated inactivating kinases. The use of molecules to stimulate the gain of tumor suppressor function through direct interaction has been little explored, and our research therefore has the potential to demonstrate the therapeutic accessibility of a novel class of targets. In addition, our research will provide important insights into how therapeutics may function by manipulating protein-protein interactions, an increasingly important class of cancer targets. For breast cancer treatment in particular, the discovery of molecules that target Rb directly will be a breakthrough towards the development of safe and effective chemotherapeutics.

5.0 CHANGES / PROBLEMS

We encountered no technical problems. We did not find an ideal lead compound from our screen of the CPCCG small molecule collection, so we are pursuing some additional approaches outlined in our proposal and described above. The overall goals and aims remain the same, and we have no significant changes to protocols to report.

6.0 PRODUCTS

Nothing to report.

7.0 PARTICIPANTS

Name:	Dr. Seth Rubin	Tyler Liban
Project Role:	PI	Graduate Student Researcher
Person Months:	1	6
Contribution to project:	Oversee project, data interpretation, and communication of results	Biophysical validation assays and preliminary crystallization of protein targets

8.0 SPECIAL REPORTING REQUIREMENTS

None

9.0 APPENDICES

None